Microbiological Studies of *Coli-aerogenes* Bacteria*

Part III. Oxidations of Glucose and Pyruvic Acid

By Hideo KATAGIRI, Tatsurokuro TOCHIKURA and Kazutami IMAI

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University

Received June 21, 1957

The oxidative degradation of glucose by *coli-aerogenes* bacteria was investigated: a remarkable yield (more than 0.5 mole per one mole of glucose) of α -ketoglutaric acid was obtained with suspensions of washed cells of the bacteria grown aerobically on glucose medium, and pyruvic acid fermentation was revealed in the presence of arsenic compounds not only with growing culture but also with the washed cells. Moreover, pyruvic acid was found to be a potent precursor of α -ketoglutaric acid.

INTRODUCTION

In the previous paper¹⁾, the authors have made investigations on the fermentation of glucose by the bacteria of *coli-aerogenes* types and it was found that when the bacteria were cultivated under aerobic conditions in replacement of usual anaerobic incubation, a large amount of α -ketoglutaric acid was obtained in place of lactic acid as the major product of fermentation. From the results of the authors' experiments, with various kinds of *coli-aerogenes* bacteria, there were also pointed out so many types of their oxidative fermentations, such as α -ketoglutaric acid-, pyruvic acid-, acetic, pyruvic (acetoine) and α -ketoglutaric acids-, acetic and α -ketoglutaric acids-fermentations and the complete oxidation of glucose into CO_2 and H_2O .

Koepsell et al., investigated the production of pyruvic and α -ketoglutaric acids in oxidative fermentations of gluconic and 2-ketogluconic acids by *Pseudomonas fluorescens* and suggested that α -ketoglutaric acid might arise from the condensation of pyruvic acid with fragments such as C_2 - or C_3 compounds pro-

duced during fermentation, since the accumulation of pyruvic acid was decreased against the increasing yield of α -ketoglutaric acid at the later stage of growing culture of the bacterium.

In the present paper, various investigations were instituted with glucose and pyruvic acid in order to ascertain the yield of α -ketoglutaric acid, and to ascertain whether pyruvic acid would be obtained in the oxidation of glucose by the bacteria of *coli-aerogenes* types, and also to ascertain whether such a three-carbon compound as pyruvic acid would be potent in the production of α -ketoglutaric acid.

EXPERIMENTAL AND RESULTS

Production of α -Ketoglutaric Acid from Glucose by Washed Cells of *E. Coli*

In the present paper experiments on the production of α -ketoglutaric acid from glucose were carried out with washed cells of *E. coli* grown aerobically on glucose medium. One hundred ml of a medium containing 2% glucose, 0.1% $(\text{NH}_4)_2\text{HPO}_4$, 0.3% $(\text{NH}_4)_2\text{SO}_4$, 0.1% K_2HPO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.2% NaCl was taken in a 500 ml shaking flask and sterilized CaCO_3 of the amount corresponding to 50% of glucose was added in a flask at the time of inoculation. The bouillon culture of *E. coli*, obtained by 1-2 days' incubation at 37°C under static conditions was used as

* A part of this paper was read before the annual meeting of the Japanese Biochemical Society (November, 1955).

1) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 210, 215 (1957).

TABLE I
YIELD OF α -KETOGLUTARIC ACID FROM GLUCOSE BY WASHED CELLS OF
E. COLI GROWN ON GLUCOSE MEDIUM

Bacterial cells (mg)	Time of incubation (hours)	Addition of CaCO_3 (mg)	Initial conc. of glucose (μM)	Glucose consumed (μM)	α -Ketoglutaric acid produced	
					(μM)	(Molar yield)
41	13	0	1000	—	228	0.23
41	13	0	1000	—	253	0.25
48	16	0	1000	—	203	0.20
63	15	0	1000	—	216	0.22
38	16	0	1000	—	310	0.31
46	11	100	1000	—	577	0.58
51	13	100	1000	—	612	0.61
43	13	100	1000	753	410	0.54
43	13	100	1000	787	312	0.40
36	15	100	1000	842	600	0.71
51	14	100	1000	1000	762	0.76

an inoculum and the flask was placed on a shaker. After incubation at 30°C for 2 days, the bacterial cells were collected by a centrifuge and washed with distilled water. The bacterial cells were then suspended in distilled water and the concentration was determined by the optical density, observed with an electrophotometer. The reaction mixtures (5–20 ml) containing 1 mM glucose, 1 mM phosphate buffer (pH: 6.2), 10 μM MgSO_4 , 0–100 mg CaCO_3 and the suspensions of washed cells, were dispensed in a 100 ml test tube or a 500 ml shaking flask, and kept aerobically on the shaker at 28–30°C. After incubation, the bacterial cells were removed by a centrifuge and then the amounts of consumed glucose and produced α -ketonic acids (α -ketoglutaric and pyruvic) were determined by the methods mentioned in the previous paper¹³.

From the results shown in Table I, it can be recognized that α -ketoglutaric acid was produced without any accumulation of pyruvic acid in the course of fermentation of glucose by the washed cells of *E. coli* grown on glucose medium and that the yields of α -ketoglutaric acid exceeded 0.5 mole (40.6 g acid from 100 g glucose) from one mole of glucose, which would often be observed with growing culture reported in the previous paper.

The fact that α -ketoglutaric acid is produced more than 0.5 mole from one mole of glucose has also been ascertained in the growing cultures of microorganisms other than *coli-aerogenes* by Koepsell et al.²³, Masuo et

al.⁴², Asai et al.⁵³ and Doi et al.⁶³. The predicted theoretical yield of α -ketoglutaric acid would be 0.5 mole per mole of consumed glucose according to the Warburg-Lipmann-Dickens pathway in which oxidation of glucose-6-phosphate to 5-phosphopentose via 6-phosphogluconate, thence, 6-phospho-2-ketogluconate has been suggested. This one-carbon degradation of the glucose molecule is considered to be repeated until one mole of triose phosphate and three moles of carbon dioxide are generated.

Although the existence of the above pathway in the bacteria belonging to *E. coli* has already been pointed out by some workers²³, the results of our experiments from which such a high yield of α -ketoglutaric acid as more than 0.5 mole per one mole of consumed glucose is ascertained, suggest that such alternative pathways relating to the Embden-Meyerhof scheme or $\text{C}_6 \rightarrow \text{C}_5 \rightarrow \text{C}_2 + \text{C}_3$ would exist besides the Warburg-Lipmann-Dickens scheme.

Conversion of α -Ketoglutaric Acid Fermentation to Pyruvic Acid Fermentation in Presence of Arsenite or Arsenate

Experiments of Growing Culture. Arsenite is a

4) E. Masuo and Y. Wakizaka, *J. Agr. Chem. Soc. Japan*, **29**, 546 (1955); *ibid.*, **29**, 550 (1955).

5) T. Asai, K. Aida, Z. Sugisaki and N. Yakeishi, *J. Gen. Appl. Microbiol.*, **1**, 308 (1955).

6) S. Doi et al., Reported at the meeting of the Agricultural Chemical Society of Japan held in 1956 in Ise City.

7) S.S. Cohen et al., *J. Biol. Chem.*, **188**, 509 (1951); *Fed. Proc.*, **10**, 173 (1951); *Biochem J.*, **55**, 23 (1953); *ibid.*, **55**, 33 (1953).

2) H.J. Koepsell, *J. Biol. Chem.*, **186**, 743 (1950).

3) H.J. Koepsell, F.H. Stodola and E.S. Sharpe, *J. Am. Chem. Soc.*, **74**, 5142 (1952).

well known inhibitor of the oxidative decarboxylation of α -ketoglutaric acid to succinic acid and carbon dioxide^{5,8,10}. In the present paper, oxidative fermentations of *E. coli* were at first, instituted on a shaker with a medium containing 10% glucose, 0.1% $(\text{NH}_4)_2\text{HPO}_4$, 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.1% K_2HPO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 5% CaCO_3 with or without Na-arsenite which was added after 1 day's fermentation, in order to ascertain what influence would be exerted by arsenite on the production of α -ketoglutaric acid from glucose with the growing culture of the bacterium.

TABLE II
INFLUENCE OF Na-ARSENITE ON OXIDATION
OF GLUCOSE IN GROWING
CULTURE OF *E. COLI*

Addition of Na-arsenite (M/l)	0	2×10^{-3}	4×10^{-3}
Glucose consumed (mM/dl)	44.0	42.6	50.5
α -Ketoglutaric acid produced (mM/dl)	20.5	16.4	15.0
(Molar yield)	0.47	0.39	0.30
Pyruvic acid produced (mM/dl)	0	12.5	25.5
(Molar yield)		0.29	0.51
Acetic acid produced (mM/dl)	0	2.34	4.84
(Molar yield)		0.05	0.09

It will be seen in Table II that such a remarkable production of pyruvic acid took place, that the maximum yield amounted about 0.51 mole per one mole glucose consumed, while the accumulation of α -ketoglutaric acid (Yield being about 0.30 mole) was considerably decreased when the bacterium was cultivated under aerobic conditions in the presence of 4×10^{-3} M Na-arsenite.

8) T.K. Walker et al., *Nature*, **168**, 1042 (1951).

9) D.J.D. Hockenhull et al., *ibid.*, **168**, 1043 (1951).

Similar experiments were carried out with the media containing 2% glucose, 0.1% $(\text{NH}_4)_2\text{HPO}_4$, 0.1% K_2HPO_4 and other mineral matters in either the presence or absence of Na-arsenate. The results of the

TABLE III
INFLUENCE OF Na-ARSENATE ON OXIDATION OF
GLUCOSE IN GROWING CULTURE OF *E. COLI*

Addition of Na-arsenate (M/l)	0	8.1×10^{-3}	2.4×10^{-2}
Glucose consumed (mM/dl)	7.87	7.60	7.87
α -Ketoglutaric acid produced (mM/dl)	4.78	3.43	1.71
(Molar yield)	0.61	0.45	0.22
Pyruvic acid produced (mM/dl)	0	2.84	6.84
(Molar yield)		0.37	0.87

experiments are given in Table III, from which pyruvic acid fermentation was again observed to have taken place, instead of α -ketoglutaric acid fermentation, with glucose medium containing Na-arsenate, although the yields of pyruvic as well as α -ketoglutaric acids varied with the concentration of arsenic compound.

Experiments with Suspensions of Washed Cells of *E. Coli*. The influences of arsenate upon oxidative fermentation of glucose were further investigated in the experiments with the washed cells of *E. coli* grown aerobically at 30°C for 2 days on 2% glucose medium in which the same mineral matters were employed as in the experiments shown in Table I. Table IV shows the results of the experiments, which were instituted as follows: the reaction mixture (7.5 ml) contained 0.5–1.0 mM phosphate buffer (pH: 6.2), 10 μM MgSO_4 , 150 mg CaCO_3 , 1 mM glucose, and the requisite amounts of washed cells and of Na-arsenate which were illustrated in the Table, fermenta-

TABLE IV
INFLUENCE OF Na-ARSENATE UPON OXIDATION OF GLUCOSE BY SUSPENSION
OF WASHED CELLS OF *E. COLI*

Exp. No.	Bacterial cells (mg)	Addition of Na-arsenate (μM)	Glucose consumed (μM)	α -Ketoglutaric acid produced		Pyruvic acid produced	
				(μM)	(Molar yield)	(μM)	(Molar yield)
Ia	43	0	842	600	0.71	0	
Ib	43	484	1000	270	0.27	702	0.70
Ic	43	648	1000	243	0.24	800	0.80
Id	43	970	1000	204	0.20	926	0.93
IIa	51	0	1000	762	0.76	0	
IIb	51	162	1000	572	0.57	270	0.27
IIc	51	162	1000	448	0.45	557	0.56
IIId	51	970	835	312	0.37	550	0.66

tions being carried out on the shaker at 30°C for 14 hours. It will be seen from Table IV that pyruvic acid was again produced remarkably from glucose by the washed cells in presence of higher concentration of Na-arsenate with which the production of α -ketoglutaric acid was extremely decreased. Therefore, the influence of arsenate upon the kinds of oxidation products obtained with washed bacterial cells was found to be the same as those observed with growing culture of the bacterium. The results of observations hitherto mentioned suggest the possibility that the action of arsenic compounds such as arsenite or arsenate would inhibit pyruvic oxidase to cause a conversion of α -ketoglutaric acid fermentation to pyruvic acid fermentation and pyruvic acid would be a potent intermediate substance in the production of α -ketoglutaric acid, since, the amount of increasing pyruvic acid were found to be nearly equivalent to those of decreasing α -ketoglutaric acid revealed by the inhibitors.

Manometric Experiments. The conventional Warburg technique was used for the determination of oxygen and carbon dioxide in the following manner: the reaction mixture (2.5 ml) was composed of 300 μ M phosphate buffer (pH: 6.0), 100 μ M glucose, 2 μ M

MgSO₄ and the washed cells (29 mg on dry basis) of *E. coli* grown on glucose medium and incubation was carried out in either the presence or absence of Na-arsenate (320 μ M) at 30°C in air. As can be seen in Fig. 1, the evolution of carbon dioxide was remarkably decreased in presence of Na-arsenate but considerable difference of oxygen uptake was not pointed out by the inhibitor, so that the R.Q. values calculated were found to be about 0.5–0.65 and about 1.0 in the presence and absence of arsenate respectively. These results, therefore, show that some portion of the oxygen consumed during aerobic decomposition of glucose would be attributed to some other reactions not involving oxidative decarboxylation. In the case of pyruvic acid fermentation, it is considered that some portion of molecular oxygen could be consumed through the reaction chain composed of diaphorase, cytochrome and cytochrome oxidase, following the coupled reaction between the reduction of coenzyme and oxidation of phosphoglyceraldehyde to phosphoglyceric acid.

Production of α -Ketoglutaric Acid from Pyruvic Acid by Washed Cells of *E. Coli*

The authors' interest has been directed to pyruvic acid which might be supposed to be an intermediate substance in the formation of α -ketoglutaric acid from glucose according to the experimental results obtained by Koepsell et al., and also by the authors. The following experiments were at first instituted in order to verify the synthesis of α -ketoglutaric acid from pyruvic acid by washed cells of *E. coli* with which the production of α -ketoglutaric acid from glucose was

FIG. 1. Effect of Na-arsenate upon Oxidation of Glucose by Washed Cells of *E. coli*.

- I. Glucose (Oxygen uptake).
- II. Glucose (Carbon dioxide evolution).
- III. Glucose + Na-arsenate (Oxygen uptake).
- IV. Glucose + Na-arsenate (Carbon dioxide evolution).

TABLE V
PRODUCTION OF α -KETOTGLUTARIC ACID FROM
PYRUVIC ACID BY WASHED CELLS OF
E. COLI GROWN ON GLUCOSE MEDIUM

Bacterial cells (mg)	Time of incubation (hours)	Pyruvic acid consumed (μ M)	α -Ketoglutaric acid produced	
			(μ M)	(Molar yield)
240	13	4000	773	0.194
240	13	2000	204	0.102
245	15	4000	1170	0.293
245	15	2000	400	0.200
200	15	4000	383	0.096
335	16	4000	216	0.054
148	13	4000	1335	0.334
145	8	2000	593	0.296
175	14	4000	450	0.113

TABLE VI
INFLUENCE OF CONCENTRATIONS OF WASHED CELLS OF *COLI-AEROGENES* UPON YIELD
OF α -KETOGLUTARIC ACID OBTAINED FROM PYRUVIC ACID

Exp. No.	Bacterial cells (mg)	Time of incubation (hours)	Pyruvic acid consumed (μ M)	α -Ketoglutaric acid produced	
				(μ M)	(Molar yield)
Ia	145	14	4000	1160	0.290
Ib	435	14	4000	493	0.123
IIa	148	14	4000	1335	0.334
IIb	444	14	4000	830	0.207

ascertained as was already mentioned. The bacterial cells were collected by a centrifuge and then suspended in distilled water after being washed. The reaction mixture (40 ml) contained 5 mM phosphate buffer (pH: 6.2), 100 μ M MgSO_4 , requisite amounts of Na-pyruvate and bacterial cells as are illustrated in Table V, and incubated aerobically on the shaker at 30°C. The results of the experiments are given in Table V, from which washed cells of *E. coli* grown aerobically on glucose medium have been found to be capable of producing α -ketoglutaric acid from Na-pyruvate. The production of α -ketoglutaric acid from pyruvic acid would necessarily involve carbon synthesis ($\text{C}_3 \rightarrow \text{C}_5$) and yet α -ketoglutaric acid was obtained with such high yields as shown in Table V. It is a well known fact that pyruvic acid is metabolized aerobically by the bacteria of *coli-aerogenes* types to acetic acid¹⁰, CO_2 , H_2O and to cell materials¹¹, namely, various reactions including acetic acid fermentation, complete oxidation (normal respiration) and oxidative assimilation would take place by the bacteria. However, from the experimental results obtained by the authors it is recognized that α -ketoglutaric acid is the major oxidation product of pyruvic acid, although the yields of α -ketoglutaric acid from pyruvate were observed to be extremely dependent upon the experimental conditions as will be seen in Table VI in which better yields of α -ketoglutaric acid were obtained at a higher ratio of pyruvic acid/bacterial cells. These experimental results correspond to those of growing cultures observed in the previous paper in which good yields of α -ketoglutaric acid from glucose were obtained at a higher ratio of carbon/nitrogen, while at a lower ratio, the yield of α -ketoglutaric acid was exceedingly

decreased.

SUMMARY

1. With washed cells of the bacteria of *coli-aerogenes* grown aerobically on glucose medium, the yield of α -ketoglutaric acid from glucose was found to exceed 0.5 mole per one mole of glucose consumed, and the mode of oxidative degradation of glucose was discussed.

2. In presence of arsenic compounds, it was observed that the oxidative fermentation of glucose with either washed cells or growing culture of *E. coli* gave rise to pyruvic acid as a major product, while the evolution of carbon dioxide was remarkably decreased and the mode of oxidation of glucose was again discussed from the standpoint of pyruvic acid fermentation.

3. It was found that α -ketoglutaric acid was obtained in a high yield (0.5–0.7 mole from two moles of pyruvic acid) when the washed cells were incubated on the shaker with a Na-pyruvate solution. According to the experimental conditions, a remarkable difference was pointed out among the yields of α -ketoglutaric acid.

This investigation has been supported in part by a Grant in Aid for Fundamental Scientific Research defrayed from the Ministry of Education, to which the authors' thanks are due.

10) J.L. Still, *Biochem. J.*, **35**, 380 (1941).

11) C.E. Clifton, *Advances in Enzym.*, **6**, 269 (1946).